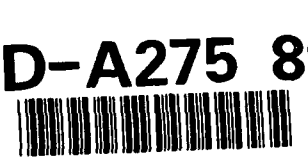

(2)

FIRST TRIANNUAL REPORT (YEAR 1)

for period September 1, 1993 to December 31, 1993

Report Date: February 2, 1994

ONR Grant No. N00014-93-J-1034
(ECU Grant #5-01071)

PRECLINICAL INVESTIGATION OF LYOPHILIZED PLATELET PREPARATIONS

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Attachment: Report from subcontract principal investigator, Marjorie S. Read, Ph.D., The University of North Carolina at Chapel Hill.

Administrative Activity:

In the first month of the grant, a new subcontract agreement was set up between ECU and UNC-CH and budgets initiated in three different departments of collaborators at ECU. We immediately hired a level one lab technician into a temporary position to begin the canine preparations at ECU; that position is now established as permanent full-time and open for hiring. All other personnel were already in place. A micro computer with 486 processor, large monitor, and color ink jet printer was purchased at ECU to perform statistics (to replace the previous 286 microcomputer which had a failed hard drive) and to run a flow cytometry analysis program with color output.

One item of equipment listed for Year 1, the Xylum Clot Signature Analyzer (an ex vivo bleeding time testing device) was not deliverable by the vendor in this reporting period because of a redesign of the commercial model. A prototype device was used previously by Don Gabriel, M.D., of UNC-CH to demonstrate the efficacy of our lyophilized platelet preparations in restoring normal hemostasis to von Willebrand's or thrombocytopenic whole blood. We plan to use the CSA in the in vivo trials of lyophilized canine platelets in dogs given a platelet function defect by extended extracorporeal bypass circulation. An agreement has now been worked out with the manufacturer to supply us with a functional prototype until the commercial devices are approved by the FDA and released for distribution (June, '94). Preliminary preparations for this type of experiment are proceeding on the assumption that the prototype CSA will be in place within a month.

Scientific Progress:

This project represents an acceleration of our overall efforts to characterize and test lyophilized platelet preparations in vivo and in vitro to prepare for clinical trials in humans as soon as possible. Both worksites (ECU and UNC-CH) have initiated SDS PAGE analysis of lyophilized platelets under various conditions to determine protein content and secretory activity of these preparations versus fresh platelets. In addition, infusions of lyophilized platelets into animal models are being pursued at both institutions (especially Specific Aims #1 and 2, see attached subcontract report).

At ECU, we have begun processing and stockpiling lyophilized canine platelets (15 preparations since Sept. 1, but with highly variable yields) to refine the method and to produce enough material to provide a massive (one-half platelet mass) transfusion in our first in vivo study of the correction of platelet function in a dog on sustained heart-lung bypass (Specific Aim #4(a) of the general proposal and workplan). We hope to complete five or more such studies in the next reporting period. Analysis of the effect of infused lyophilized platelets in the experimental dogs will be based on several versions of bleeding time assessment, including the prototype Xylum CSA device discussed above and an ear bleeding time test by the Ivy method. Baseline studies of dogs on pump for other protocols have already been initiated.

SDS PAGE high resolution analysis has been run at ECU on several para-platelet preps to look for integrity of internal and external glycoproteins and proteins involved in regulation of the cytoskeleton or function of lyophilized platelets. The current work is leading up to experiments to evaluate the ability of lyophilized platelets to phosphorylate structural and regulatory proteins in response to physiologic stimulation (see Specific Aim #5). We have seen thus far that only a small fraction of the total platelet protein is irreversibly cross-linked by the stabilization and freeze-drying process and won't enter the resolving gels. We have determined that it is feasible to proceed to analysis of neoantigen binding of proteins such as thrombospondin to the insoluble cytoskeleton as a signal of activation. Furthermore, we have performed radioimmunoassay of Thromboxane B_2 in two batches of samples before and after Baumgartner perfusion experiments with lyophilized human platelets circulated over everted canine arterial vessel strips. The results show that TxB_2 is present in very high amounts just upon rehydration of freeze-dried para-platelets (up to 40,000 picograms per 0.1 mL supnt), and that during the Baumgartner experiments TxB_2 levels rise to 3,000 pg/0.1 mL in para-platelet preps, but not others. Fourteen preparations have been tested in this way, along with three liquid platelet concentrates; fresh controls were run in each experiment. These data are being prepared for publication in combination with the adhesion and flow cytometry data gathered under Navy grant N00014-92-J-1244.

With the above information we are pleased to report that the specific aims are well underway and generating meaningful data. The next progress report should include the results of multiple "n" in each of the experimental designs.

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Report Period September 1, 1993-December 31, 1993
University of North Carolina at Chapel Hill

Contract: UNC/ECU
Grant No. N-000-14-93-1034
The Office of Naval Research
Department of the Navy

Performance Site: University of North Carolina at Chapel Hill
Principal Investigator: Marjorie S. Read, Ph.D.
Co-PI: Robert Reddick, MD
Submitted: January 31, 1994

Studies conducted during the reporting period October 1993-December 1993

<u>The biochemical and immunological characterization of human and canine platelets that have undergone the freeze-drying process (Specific aim #1)</u>

The characterization of rehydrated human and canine platelets is underway. We have begun the characterization of rehydrated platelets using SDS-PAGE and immunoblotting of human and canine platelet preparations. Rehydrated and fresh platelets were lysed using a freeze-thaw protocol or using a detergent such as Triton X-100. We have found that, as expected, the rehydrated platelets are not lysed with the facility of fresh platelets. SDS-PAGE was performed on the platelet lysates and silver staining was used to visualize the proteins. Our results suggest that rehydrated human platelet lysates have a similar but nonidentical protein profile on SDS-PAGE when compared to fresh human platelet lysates. Similar studies are currently underway using canine fresh and rehydrated platelets.

We have also used SDS-PAGE on 'releasates' of human rehydrated and fresh platelets. Releasates were generated by incubating the fresh or rehydrated platelet in buffer for a finite period of time, allowing the release of proteins from the platelet into the supernatant, or releasate. We have found that although the releasates from rehydrated and fresh human platelets contain many proteins in common, there are at least two protein bands present in the rehydrated human platelet releasate which are absent in the fresh human platelet releasate. We have also found that the quantity of protein released from the rehydrated platelets is lower than that released from the fresh platelets. These findings suggest that rehydrated human platelets are capable of protein secretion, although in a less dramatic manner than that seen in fresh human platelets. Similar studies are being performed on rehydrated and fresh canine platelets.

Immunoblotting was also performed on the lysates of fresh and rehydrated platelets. A commercially available antibody to human platelet glycoprotein IIbIIIa (AMAC, Inc., Westbrook, ME) was used to probe immunoblots of rehydrated and fresh human platelet lysates. Our results indicate that, like fresh platelet lysates, rehydrated platelet lysates exhibit reactivity with antibodies to GPIIbIIIa on immunoblots. We are currently testing other commercially available antibodies to thrombospondin (AMAC, Inc., Westbrook, ME), GPIb (DAKO, Denmark), fibrinogen (Calbiochem Corp., San Diego, CA) and fibronectin (Calbiochem Corp., San Diego, CA) on immunoblots of rehydrated platelet lysates to further characterize rehydrated human platelets. We have also probed immunoblots of rehydrated platelet preparations with antibodies that we have generated to fixed and fresh human and canine platelets with the generous support of Navy grant #N0014-92-J-1244. Preliminary studies using these antibodies suggest that there are some changes in the immunological reactivity of platelet surface receptors in rehydrated platelets. Using an antibody generated to rehydrated human platelets as a probe for an immunoblot of rehydrated and fresh human platelet lysates, we have found reactivity with specific bands in the rehydrated platelet lysate, but not with the fresh platelet lysate. We will use these antibodies to continue the characterization of the rehydrated human and canine platelets.

The effects of multiple infusions of rehydrated platelets in the canine animal model (Specific aim #2)

In our studies on the effects of multiple transfusions of rehydrated platelets, we infused a normal adult beagle 7 times with rehydrated canine platelets which had been prepared with canine serum albumin. Each infusate was a mixture of rehydrated platelets prepared from 3 unrelated dogs. Blood samples were collected pre- and post-transfusion from the dog and tested for RBC, WBC, platelets, diagnostic multi-chem profile, electrolytes, general chemistries and enzymes. Most samples fell within normal range for the tests performed. An shallow increase in the WBC was noted after the fifth infusion, with a return to normal levels within 24 hours. This increase in WBC is thought to be attributable to contamination in the platelet preparation prior to infusion. A decrease in the neutrophil count with a concomitant increase in lymphocyte count was noted after the sixth infusion. This change in the WBC differential might be attributed to sampling error. No other significant changes in blood profiles were noted. The dog had no other visible responses to the infusions, such as weakness and lethargy, which have been noted in previous experiments. The respiration and temperature of the dog remained normal throughout the infusion experiment.